BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Exhibits

Exhibit A. Sastry *et al.*, *Catalytic Antibodies*, 1991, Ciba Foundation, 159, pp. 145-155

Screening combinatorial antibody libraries for catalytic acyl transfer reactions

Lakshmi Sastry, Monica Mubaraki, Kim D. Janda, Steve J. Benkovic and Richard A. Lerner

Department of Molecular Biology & Chemistry, Research Institute of Scripps Clinic, La Jolla, CA 92037, USA

Abstract. A bacteriophage λ vector system for the expression of Fab fragments from the mouse antibody repertoire in *Escherichia coli* has been described. We have used this system to generate a catalytic antibody from a combinatorial antibody library. Monoclonal antibody 43C9 was raised against a transition state analogue of the hydrolysis of carboxyamide. mRNA from hybridoma cells expressing this antibody was cloned into phage λ and clones that expressed the mRNA for either the heavy or the light chain of the antibody were isolated. These individual libraries were then crossed to generate a combinatorial library in which clones coexpressed the heavy and light chains. This library was screened for antibodies/Fab fragments that bound to the original antigen with high affinity. DNA sequencing showed that these fragments were the same as those in antibody 43C9. Three different clones were found to catalyse the hydrolysis of carboxyamide. More efficient expression vectors and improved screening techniques should lead to the isolation of many more catalytic antibodies from combinatorial antibody libraries.

1991 Catalytic antibodies. Wiley, Chichester (Ciba Foundation Symposium 159) p 145–155

Monoclonal antibodies are used extensively in various fields of biology and medicine. Some important applications include the investigation of cellular mechanisms, the isolation of interferons, cancer research, clinical diagnosis and gene product analysis. The generation of monoclonal antibodies with specific catalytic functions is an emerging technology that combines the high specificities of antibodies with chemical catalysis. A number of reactions have been successfully catalysed by monoclonal antibodies (for review see Lerner & Benkovic 1988). The production of homogeneous antibodies for catalysis is entirely dependent on the hybridoma technology; but this is an inefficient method

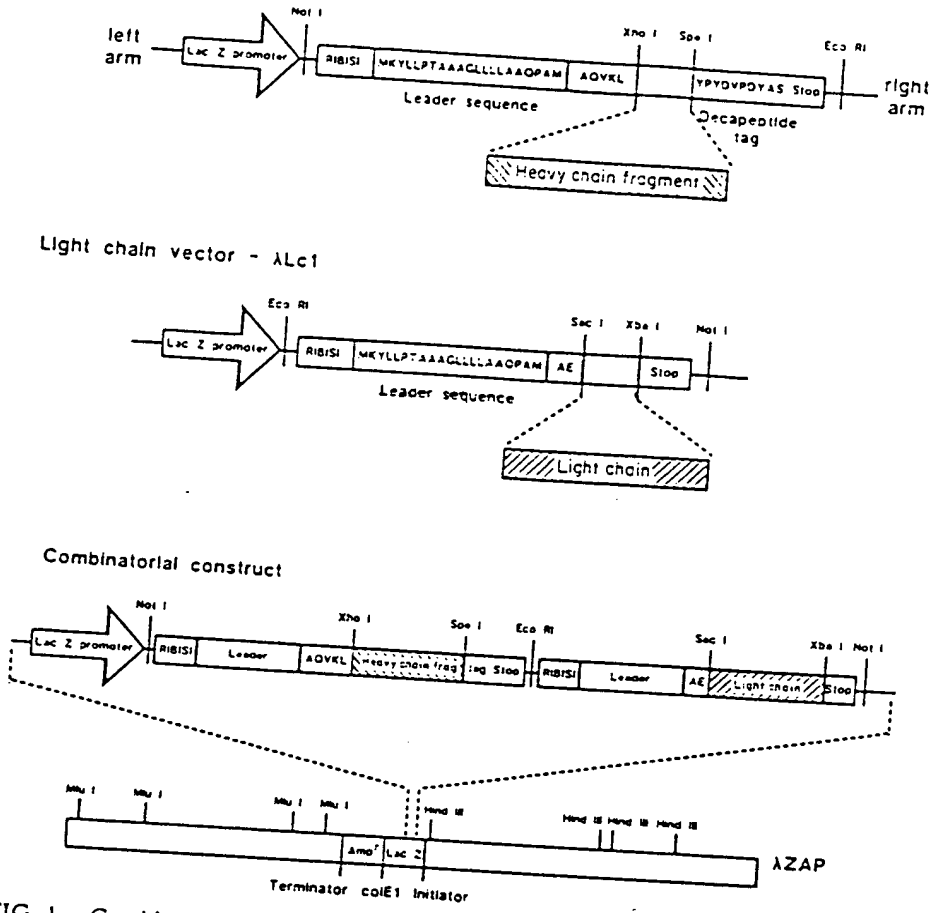

FIG. 1. Combinatorial bacteriophage λ vector system for expression of Fab antibody fragments. The LC1 vector is for cloning PCR products of mRNAs that code for κ light chains; the HC2 vector is for cloning PCR products of mRNAs coding for heavy chain Fd sequences. The combinatorial constructs that can express Fab fragments are generated by cutting light and heavy chain DNA at the antisymmetric *Eco*R1 site of each vector, followed by religation of the resulting arms.

for surveying the immunological repertoire and limits the number of catalysts that can be obtained. We have developed a system using bacteriophage λ to clone and express a combinatorial library of Fab fragments of the mouse antibody repertoire in *Escherichia coli* (Fig. 1) (Sastry et al 1989, Huse et al 1989). The system allows rapid and easy identification of monoclonal Fab fragments in a form suitable for genetic manipulation. However, it remains to be shown that such combinatorial libraries can be used to produce catalytic Fab

fr: .ents. In this paper we demonstrate the generation of a catalytic antibody from a combinatorial antibody library.

Using the λ phage system we generated an Fab combinatorial library from the spleen of a mouse immunized with phosphonamidate 1 (NPN), a transition state analogue for the hydrolysis of carboxyamide substrate 2 (Fig. 2). Screening the library with the antigen, NPN, linked to bovine serum albumin (NPN-BSA) resulted in the identification of a number of Fab fragments that bound to the antigen in a competitive manner. To find efficient catalysts for the hydrolysis of the nitroanilide 2, we screened the Fab combinatorial library directly for catalysis. The induced phage libraries were incubated with nitrocellulose filters saturated with the substrate, or the substrate was added directly to agar containing cells infected with the phage before they were poured onto a plate. Unfortunately, these approaches were unsuccessful because of the chemical nature of the reaction as well as the limited amount of Fab that is secreted by the phage molecules. It has previously been observed that catalysis of hydrolysis of the amide 2 occurs at 37 °C with high concentrations of an antibody (Janda et al 1988).

The high concentrations of antibody required for catalysis are difficult to achieve directly on the phage surface. Also, the product of the hydrolysis, *p*-nitroaniline, is diffusible and is hard to observe either directly on phage

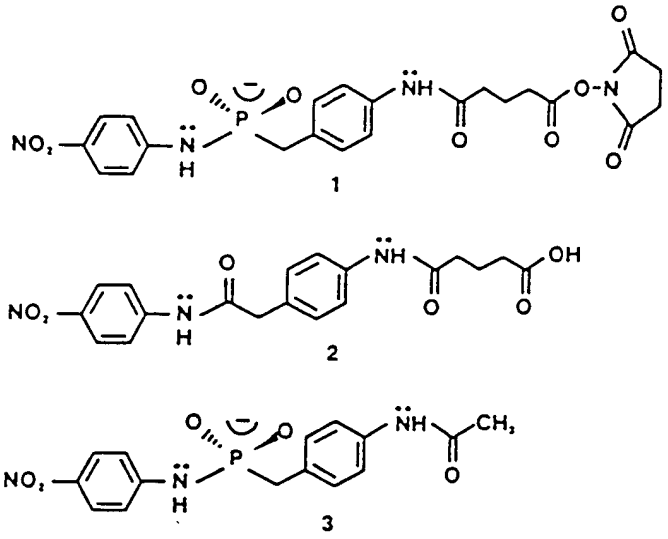

FIG. 2. The transition state analogue phosphonamidate 1 (NPN) which was used to induce antibodies that hydrolyse the carboxyamide substrate 2. The phosphonamidate functionality mimics the stereoelectronic features of the transition state for hydrolysis of the amide bond. The transition state analogue 3 is an inhibitor of the reaction.

plaques or on nitrocellulose filters. Because of these practical limitations, we decided to screen initially for Fab fragments that bound to NPN and then for those that showed catalytic activity. As an essential first step, we cloned and expressed a monoclonal antibody (43C9) that catalyses the amide hydrolysis in the phage system (Janda et al 1988). Besides being an internal control, the expression of the monoclonal antibody in phage also allows the study of its structure and mechanism of catalysis. Mutagenesis and chain-exchange experiments can be easily performed on the cloned antibody to improve its catalytic activity.

Methods

Total RNA from 10^7 43C9 hybridoma cells was isolated as described (Chomczynski & Sacchi 1987). The mRNAs were purified on an oligo dT column, then amplified using the polymerase chain reaction to obtain separate pools of heavy and light chain DNA (Sastry et al 1989, Huse et al 1989). Amplification of heavy chain DNA was performed with eight different 5′ primers and a 3′ primer specific for the IgG2b isotype. Light chain DNA was similarly amplified with five 5′ primers and a κ chain-specific 3′ primer. Heavy and light chain libraries were generated in phage λ and crossed to obtain an Fab combinatorial library (Huse et al 1989). This library was then screened with NPN-BSA labelled with ^{125}I and Fab fragments that bound the antigen were identified (Huse et al 1989). These Fab fragments were excised using helper phage (M12 mp8) and McBlue cells and plated on LB/ampicillin plates (Short et al 1988). Colonies on the plates represented the excised plasmid carrying the cloned heavy and light chain pieces.

Individual clones were grown up and their protein products isolated using an affinity column made from anti-$(Fab')_2$ coupled to Sepharose beads. Purified Fab was dialysed for 4–6 hours against ATE (Aces, Tris, ethanolamine) buffer, pH 9.0, concentrated to 1–3 μM solution, and used for catalysis. Catalysis was performed at 37 °C in ATE buffer at pH 9.0 with the 1–3 μM Fab solution and a saturating amount (1 mM) of substrate 2. Sequencing of the positive clones was as described by Sanger et al (1977).

Results

PCR amplification of heavy and light chain DNA resulted in bands of about 700 bp as analysed by agarose gel electrophoresis. A number of different primers were used for amplification from the hybridoma cells, because these may contain other non-functional heavy or light chains and restricted amplification may result in the cloning and expression of the wrong chains. To avoid this problem, we pooled the amplified DNA from the heavy and the light chains, then cloned each pooled fraction into the expression vector. Cloning of heavy chains resulted in 2×10^6 recombinants; the light chain library contained 5×10^5 recombinants.

ening of the heavy chain recombinants with an antibody raised against a conserved 10 amino acid sequence in the heavy chain showed that 90% of these were expressing the decapeptide and therefore the heavy chain. Anti-κ antibody screening of the light chain library indicated that 60% of the clones were expressing κ light chains. The combinatorial library consisting of 2×10^7 recombinants was screened with the anti-decapeptide and anti-κ antibodies; 65% of the clones coexpressed heavy and light chains.

The Fab library (3000 plaques/plate) was then screened with iodinated NPN-BSA and positive clones were identified after a three-day exposure. Fragments that bound the antigen (binders) were identified at a frequency of 1/200; this relatively low frequency may be due to the presence of non-functional heavy and light chains in the Fab library. Ideally, amplification of the hybridoma RNA with specific 5′ heavy and light chains should generate Fab fragments that bind at a much higher frequency.

The DNA sequences of the binders were obtained to identify the clone that exactly represents the monoclonal catalytic antibody 43C9. Comparison of the light chain deduced N-terminal amino acid sequence of antibody 43C9 and the deduced amino acid sequences of ten of the binders indicated that five of the clones (8a11, 8a12, 8a1, 7a2, 7a4) had the correct light chain. Three of these clones (8a1, 8a11, 8a12) were identical and differed from each of the other two (7a2, 7a4) by a single amino acid in the framework region. All the clones had the same heavy chain sequence; comparison with the N-terminal sequence of the authentic antibody was not possible because its N-terminus is blocked.

Purified Fab from each of the ten clones described above was assayed for catalytic activity; 8a11, 7a2 and 7a1 hydrolysed amide 2 at a rate clearly above the background rate (Fig. 3). The reaction was inhibited completely by the addition of transition state analogue 3, 20 μM. This indicated that the observed catalysis was exclusively due to the Fab. SDS-PAGE of the catalytic recombinant Fabs showed a single species at 50 kDa. Reducing conditions gave a doublet at 25 kDa, indicative of a single pure Fab fragment. Because of the limited amount of Fab produced in our system, detailed kinetic analysis has not been possible. Overexpression of the catalytic Fab is currently being sought, to facilitate the kinetic studies.

Discussion

The bacteriophage λ vector system developed for the expression of Fab fragments is ideally suited for studying the structure and mechanism of any desired monoclonal antibody. We have successfully expressed a monoclonal catalytic Fab in this system and have shown that it retains the ability to catalyse a specific amide hydrolytic reaction.

Future studies will be aimed at identifying more binders from the library which also display catalytic activity. The success of these will hinge upon our ability

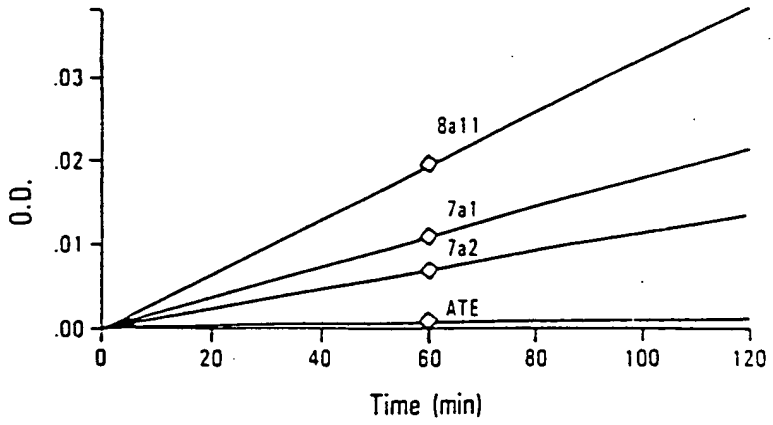

FIG. 3. Hydrolysis of carboxyamide 2 by Fab clones 8a11, 7a2 and 7a1. Hydrolysis was carried out at 37 °C with 2 μM antibody, 1 mM substrate in ATE buffer, pH 9.0. The differences in the observed rates seen for each clone probably reflect inaccuracies in protein concentration determination rather than clone differences. The background hydrolysis was measured with the substrate alone; in all cases the reaction was monitored at 405 nm.

to obtain a better system for expressing the protein, possibly utilizing Summer's baculovirus system (Smith et al 1983). More efficient screening for catalytic antibodies might be achieved via a genetic selection process.

Finally, a general solution to the antibody catalysis of a peptide bond may be obtained using the phage technology presented. Recently, we have constructed a single chain antibody with a coordination site for metals (Iverson et al 1990). When this site is incorporated into the light chain of an Fab fragment, a bound metal ion could act as a hydrolytic cofactor when properly aligned with a heavy chain which binds a small peptide sequence. The possibility of such a reaction appears remote; however, by taking advantage of the large numbers and combinations available through the combinatorial library, opportunities for success are within reach.

References

Chomczynski P, Sacchi N 1987 The calcium-sensitive dye arsenazo III inhibits calcium transport and ATP hydrolysis by the sarcoplasmic reticulum calcium pump. Anal Biochem 162:156–159

Huse WD, Sastry L, Iverson SA et al 1989 Expression of the antibody repertoire in phage λ. Science (Wash DC) 246:1275–1281

Iverson BL, Iverson SA, Roberts VA et al 1990 Metalloantibodies. Science (Wash DC) 249:659–662

Janda KD, Schloeder D, Benkovic SJ, Lerner RA 1988 Induction of an antibody that catalyses the hydrolysis of an amide bond. Science (Wash DC) 241:1188–1191

Lerner RA, Benkovic SJ 1988 Principles of antibody catalysis. Bioessays 9:107–112
Sanger F, Nicklen S, Coulson AR 1977 DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467
Sastry L, Alting-Mees M, Huse WD et al 1989 Cloning of the immunological repertoire in *Escherichia coli* for generation of monoclonal catalytic antibodies: construction of a heavy chain variable region-specific cDNA library. Proc Natl Acad Sci USA 86:5728–5732
Short JM, Fernandez JM, Sorge JA, Huse WD 1988 Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. Nucleic Acids Res 16:7583–7600
Smith GE, Summers MD, Fraser MJ 1983 Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol Cell Biol 3:2156–2165

DISCUSSION

Hansen: The exact placing of an amino acid is critical for enzyme catalysis. Do you have a sense, perhaps from Sargeson's work (Buckingham et al 1970), of how precise one has to be in orienting a carbonyl group near the metal ion to see catalysis?

Lerner: I don't know. They were basically looking at an intramolecular situation, because the substrate was directly bound to an open site on the coordination complex.

Martin: Another structural detail to consider is the geometry of the antibody's metal-binding ligands. A slight difference in the relative positions of metal-binding amino acid side chains could have a dramatic effect on the catalytic efficiency of the antibody. The coordination geometry of metals in natural enzymes is often distorted: for example, the tetrahedral geometry of cobalt-substituted carboxypeptidase A is markedly irregular compared to simple tetrahedral complexes of cobalt such as cobalt tetrachloride. In their entatic state hypothesis, Vallee & Williams (1968) proposed that the distorted coordination geometry is a critical feature of metalloenzymes in that it causes the metal to be unusually reactive—in their terms 'poised for catalysis'.

Lerner: Isn't that flying in the face of results from a great number of coordination complex experiments?

Jencks: Ground state strain of that kind can change the properties of the ions and the ligands and the metal, certainly; but to say that the ground state strain or distortion directly influences the transition state is wrong. It may provide a system which has a proper pK or oxidation potential or whatever, that will lead to a transition state more readily, and this might be done better with another metal that has a different size and a different potential, but it doesn't relate directly to the stability of the transition state.

Martin: If the enzyme or antibody binds a metal with tetrahedral geometry, say by three amino acid side chains and a reactive water molecule, the effect of the distorted coordination geometry might be to fine tune the pK of the metal-bound water molecule, thereby making it more reactive.